(54) Title: RECOMBINANT 3F8-TYPE ANTIBODIES

(57) Abstract

This invention comprises a series of recombinant monoclonal 3F8-type antibodies including recombinant murine and murine-human chimeric 3F8-type antibodies of various isotypes. The invention also includes DNA sequences encoding the murine and chimeric 3F8-type antibodies, DNA sequences encoding the antigen-binding region of 3F8, and DNA sequences encoding the complementarity determining regions of 3F8. The invention further provides methods for producing recombinant 3F8-type antibodies and pharmaceutical compositions containing 3F8-type antibodies.

l

RECOMBINANT 3F8-TYPE ANTIBODIES

5   This invention relates to a series of recombinant monoclonal
antibodies: to recombinant murine monoclonal 3F8 and to murine-
human chimeric forms of recombinant 3F8, and to uses thereof.

BACKGROUND OF THE INVENTION

10   The discovery of hybridoma technology in the mid 1970s
enabled physicians and scientists to consider the potential of
monoclonal antibodies as anti-cancer therapeutics. Although a
plethora of candidate monoclonal antibodies have been studied for
a number of malignancies, very few of these have been demonstrat-
15   ed to have imaging or therapeutic efficacy. The difficulties
encountered in developing monoclonal antibodies as imaging and
therapeutic agents for cancer indications are due to specific
properties of each antibody, the respective antigenic epitope,
the specific cancer and the patient.

20   Moreover, murine monoclonal antibodies are typically immuno-
genic in humans. A human anti-mouse antibody (HAMA) response
precludes further administration of therapeutic antibody since
HAMA effectively clears the therapeutic from the blood stream.
In addition, the intrinsic half-life of a murine antibody in a
25   human is shorter than that of a human antibody, and the Fc region
of a given murine antibody may ineffectively mediate necessary
human effector functions such as complement fixation and
antibody-dependent cellular cytotoxicity (ADCC).

Therefore, genetic engineering has been employed to make
30   certain rodent monoclonal antibodies, generated by hybridoma
technology, more human in character, enabling administration of
a functional antibody at doses which are stable and nontoxic.
Although recombinant antibodies have only recently become
available, preliminary clinical studies suggest that human
35   derivatives of specific murine monoclonals have less immunogenic-
ity and greater stability and can be efficacious.

Because of the wide range of canc rs which continue to have
high mortalities, it is desirable to expand the available
treatment modalities as widely as possible. Since a group of

patients may not share the same set of tumour surface markers on the surface of their cancer cells, production of a variety of therapeutic chimeric and humanized murine monoclonals that will bind to a particular tumour's surface markers would be beneficial

5   and could enable killing of the cells, via Fc-activation of cytotoxic immune mechanisms or conjugated toxic molecules.

SUMMARY OF THE INVENTION

The present invention provides a series of recombinantly-

10   produced 3F8-type monoclonal antibodies which are substantially free from murine proteins.

The invention also includes DNA sequences encoding the heavy and light chains of 3F8-type monoclonal antibodies, in addition to DNA sequences encoding the antigen binding site of 3F8-type

15   antibodies. Also included are DNA sequences encoding the complementarity determining region of 3F8-type antibodies.

The invention further provides a recombinantly-produced 3F8-. type chimeric antibody characterized by a light chain containing the complementarity determining regions of murine 3F8 light

20   chain, a heavy chain containing the complementarity determining regions of murine 3F8 heavy chain, the absence of the murine constant region, binding to the G_{D2} antigen, and the absence of substantial binding to the G_{D3} antigen.

Further embodiments of the invention include processes for

25   producing recombinant 3F8-type monoclonal antibodies and pharmaceutical compositions containing recombinant 3F8-type monoclonal antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

30   Figure 1a and b show the nucleotide/amino acid sequences for murine 3F8 light and heavy chain cDNAs, respectively.

Figure 2 is the nucleotide/amino acid sequence of the chimeric 3F8 V_LJ-human C_κ cDNA hybrid.

Figure 3 is the nucleotide/amino acid sequence of the

35   chimeric 3F8 V_HDJ-human $C_{\gamma 1}$ cDNA hybrid.

Figure 4 is the nucleotide/amino acid sequence of the chimeric 3F8 V_LJ-human C_κ cDNA/genomic hybrid.

3

Figure 5 is the nucleotide/amino acid sequence of the chimeric 3F8 V_HDJ-human $C_{\gamma2}$ cDNA hybrid.

Figure 6 is the nucleotide/amino acid sequence of the chimeric 3F8 V_HDJ-human $C_{\gamma3}$ cDNA/genomic hybrid.

5   Figure 7 is the nucleotide/amino acid sequence of the chimeric 3F8 V_HDJ-human $C_{\gamma4}$ cDNA/genomic hybrid.

DETAILED DESCRIPTION OF THE INVENTION

As defined in the present invention, "recombinantly-produced
10   3F8-type antibodies" are antibodies which possess at least one of the complementarity determining regions (CDRs) of native (murine monoclonal) 3F8, and which bind to antigen G_{D2} but do not bind substantially to the G_{D3} antigen. The CDRs of native 3F8 are encoded by nucleotides 134-166, 212-232 and 329-346 of Figure
15   1a, and by nucleotides 152-166, 209-256 and 353-385 of Figure 1b. Preferably, the 3F8-type antibody of the present invention contains at least one of the native 3F8 CDRs. The 3F8-type antibody of the present invention may also contain two of the native 3F8 CDRs. Another preferred embodiment of the 3F8-type
20   antibody contains at least three of the native 3F8 CDRs. In another preferred embodiment, the 3F8-type antibody contains at least four of the native 3F8 CDRs. More preferably the 3F8-type antibody contains at least five of the native 3F8 CDRs. Most preferably, the 3F8-type antibody of the present invention
25   contains six of the native 3F8 CDRs.

Recombinant 3F8-type antibodies have been produced in accordance with the present invention using a variety of cloning and gene expression techniques as indicated in the discussion and examples presented below. The cDNAs encoding both protein chains
30   of 3F8 were cloned, and the CDRs of native 3F8 were determined. Recombinant murine 3F8-type antibody may be produced in accordance with the present invention by transfection of mammalian cells with appropriate expression vectors as described in Example III.

35   Murin -human chimeric 3F8 cDNA or cDNA/genomic hybrids were generated in accordance with the present invention, in which the murine constant regions were replaced with human constant

regions, and expressed in mammalian cells. Expression of the chimeric 3F8 was optimized in accordance with the present invention by modifying the chimeric cDNA to more closely resemble a genomic construct. The isotype of the chimeric 3F8-type antibody may be changed in accordance with the present invention by substituting sequences encoding other human constant regions, e.g., the human $\gamma 2$, $\gamma 3$, and $\gamma 4$ constant regions.

Any mammalian cell may be used to establish cell lines that stably produce the recombinant 3F8-type antibodies of the present invention. Examples of suitable cell lines include but are not limited to Chinese hamster ovary (CHO) cells, COS monkey kidney cells, HeLa cells, myeloma cells, transformed B cells, hybridoma cells and other mammalian cells known in the art. In a preferred embodiment, CHO cells were used for expression of chimeric 3F8-type antibodies.

Chimeric murine/human genes constructed in accordance with the present invention comprise murine V_LJ or murine V_HDJ DNA sequences joined to human kappa constant (C_κ) or human gamma 1,2,3 or 4 constant ($C_{\gamma 1,2,3,4}$) region DNA sequences, respectively. The chimeric genes contain a precise join of the last codon of a murine J gene to the first codon of a human C region, including the codon generated by splicing of a J gene's 5' donor junction and C gene's 3' acceptor junction. The human constant regions have replaced nucleotides 380-746 of Figure 1a (light chain) and nucleotides 422-1462 of Figure 1b (heavy chain) which correspond to the murine constant region sequences.

In accordance with the present invention, active recombinant 3F8-type antibodies may be purified from conditioned media using chromatographic methods on cation exchange resin, such as S-Sepharose, followed by Sepharose-Protein A. Additional purification, concentration and, if required, buffer and exchange may be obtained by chromatography on an anion exchanger, such as Q-Sepharose, repeating the cation exchanger, such as S-Sepharose and gel filtration using, for instance, Sephacryl S300. The chimeric 3F8-type antibody can be further purified by other standard chromatographic methods, including hydrophobic interaction, mixed mode, anion exchange, and hydroxyapatite chromatogra-

phy.

The 3F8-type antibodies of the present invention mediate the
in vitro cytotoxicity of target cells that express the G_{D2}
antigen on the cell surface by either peripheral blood lympho-
cytes (or mononuclear cells), complement, neutrophils, or M-CSF
cultured monocytes. Recombinant murine 3F8 and chimeric 3F8
IgG1κ are two examples of 3F8-type antibodies which mediate this
cytotoxicity.

Pharmaceutical compositions containing the recombinant 3F8-
type antibody of the present invention may be for the in vivo
imaging and treatment of mammals by physicians in a variety of
cancers.   Some of these conditions include neuroblastoma,
melanoma, small cell lung carcinoma, and certain sarcomas.
Recombinant 3F8-type antibody is especially useful in treatment
of cancer or metastatic conditions which have proven recalcitrant
to conventional treatment.

The pharmaceutical compositions of the present invention may
also contain pharmaceutically acceptable carriers, diluents,
fillers, salts, buffers, stabilizers, and/or other materials well
known in the art. The term "pharmaceutically acceptable" means
a sterile, non-pyrogenic, non-toxic material that does not
interfere with the effectiveness of the biological activity of
the recombinant 3F8-type antibody. The characteristics of the
carrier or other material will depend on the route of administra-
tion.

A preferred pharmaceutical composition for intravenous
injection should contain, in addition to recombinant 3F8-type
antibody, an isotonic vehicle such as Sodium Chloride Injection,
Ringer's Injection, Dextrose Injection, Dextrose and Sodium
Chloride Injection, Lactated Ringer's Injection, or other vehicle
as known in the art. The pharmaceutical composition according
to the present invention may also contain stabilizers, preserva-
tives, buffers, antioxidants, or other additives known to those
of skill in the art.

Administration of the recombinant 3F8-type antibody of the
present invention can be carried out in a variety of conventional
ways. Intravenous administration to the patient is preferred,

6

wherein the recombinant 3F8-type antibody of the invention will
be in the form of pyrogen-free, parenterally acceptable aqueous
solutions.

    The amount of recombinant 3F8-type antibody in the pharma-
5   ceutical composition of the present invention will depend upon
the nature and severity of the condition being treated, and on
the nature of prior treatments which the patient has undergone.
Ultimately, the attending physician will decide the amount of
recombinant 3F8-type antibody with which to treat each individual
10   patient. It is contemplated that the various pharmaceutical
compositions of the present invention should contain about 0.1
μg to about 100 mg of recombinant 3F8-type antibody per kg body
weight.

    In practicing the method of treatment of this invention, a
15   therapeutically effective amount of recombinant 3F8-type antibody
is administered to a mammal having a cancer or metastases. The
term "therapeutically effective amount" means the total amount
of each active component of the method or composition that is
sufficient to show a meaningful patient benefit, i.e., healing
20   of chronic conditions or increase in rate of healing. When
applied to an individual active ingredient, administered alone,
the term refers to that ingredient alone. When applied to a
combination, the term refers to combined amounts of the active
ingredients that result in the therapeutic effect, whether
25   administered in combination, serially or simultaneously. A
therapeutically effective dose of the recombinant 3F8-type
antibody of this invention is contemplated to be in the range of
about 0.1 μg to about 100 mg per kg body weight per application.
The duration of intravenous therapy using the pharmaceutical
30   composition of the present invention will vary, depending on the
severity of the disease being treated and the condition and
potential idiosyncratic response of each individual patient. It
is contemplated that the duration of each application of the
recombinant 3F8-type antibody will be in the range of 0.5 to 24
35   hours of continuous intravenous administration. Ultimately the
attending physician will decide on the appropriate duration of
intravenous therapy using the pharmaceutical composition of the

7

present invention.

Recombinant 3F8-type antibody may optionally be used in combination with certain cytokines, lymphokines, or other hemato-poietic factors such as M-CSF, GM-CSF, NKSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, G-CSF, Meg-CSF and erythropoietin to treat metastatic conditions. It is contemplated that these cytokines, lymphokines and hematopoietic factors will serve to augment the power of the immune system, thereby synergizing with the recombinant 3F8 to act more efficiently, promoting the killing of the 3F8-targeted tumour cells. For example, the recombinant 3F8-type antibody may be efficaciously combined with a recombinant cytokine, such as M-CSF [G. Wong et al., Science 235, 1504-1508 (1987); U.S. 4,879,227 (Nov.7, 1989); U.S. 4,868,119 (Sep. 19, 1989); WO87/06954] and GM-CSF [G. Wong et al., Science 228, 810-815 (1985); WO86/00639] as well as NKSF [M. Kobayashi et al., J. Exp. Med. 170, 827-845 (1989); WO90/05147], G-CSF and IL-2. The recombinant 3F8-type antibodies of the present invention may additionally be co-administered with M-CSF and/or GM-CSF in accordance with WO 88/06452.

Example 1 - Isolation of Murine cDNAs that Encode the 3F8 Light and Heavy Chain Proteins

In accordance with the present invention, the cDNAs coding for the light and heavy chains of native murine monoclonal antibody 3F8 (mab 3F8) were cloned. The native murine antibody is an IgG3κ. Total RNA and then polyA+ RNA were purified from the hybridoma which secretes native murine 3F8 by conventional methods [T. Maniatis et al., Molecular Cloning: A Laboratory Manual, New York, Cold Spring Harbor Laboratory Press (1982)].

The polyA+ fraction was used as template for the synthesis of double stranded cDNA by methods known to those skilled in the art. Specific primers, KAP2PCR (TAGAGTCGACGTGGCGTCTCAGGACCTTTG TCT; all oligonucleotide sequences are 5' to 3') and PCRG32 (TAGAGTCGACCCGAGGAATGGCTAGGTGCTGTT), that contain a SalI restriction site and nucleotid s complementary to sequence 3' to the murine kappa and gamma3 constant regions, respectively, were

8

used separately to prime first-strand cDNA synthesis by AMV reverse transcriptase.

Double-stranded cDNA, with ends rendered flush by T4 DNA polymerase, was ligated to EcoR1 adaptors and then purified from excess adaptors by gel exclusion chromatography. The ends of the cDNA were then phosphorylated and ligated to pUC18 plasmid that had been incubated sequentially with EcoR1 endonuclease and calf intestine alkaline phosphatase. Competent DH5α cells (Bethesda Research Laboratories, BRL) were incubated with the ligation product and then plated in the presence of ampicillin. Filter lifts of ampicillin-resistant transformants were probed with radiolabeled oligonucleotides, either MUSKAP5 (CAGTTGGTGCAGCAT CAGCC) or GAM35 (AGATGGGGCTGTTGTTGTAG), which are complementary to the 5' ends of the murine kappa and gamma3 constant regions, respectively.

Plasmid DNA minipreps were prepared from each enriched library and several were used as templates for dideoxy-sequencing of the 3' V(D)J regions. The sequencing primers were PCRKAP3 (ATGTTAACTGCTCACTGGATGGTG) and GAM33MUS (GGATCCAGATGTGTCACTGCAG CCA) and are complementary to sequences at the 5' end of the murine kappa and gamma3 constant regions, respectively. This analysis indicated that only one population of V_LJ- and one population of V_HDJ-containing cDNAs were derived from the murine 3F8 hybridoma RNA and that these should code for the two chains of the tetrameric 3F8 monoclonal antibody.

The complete V_LJ and V_HDJ nucleotide sequences were derived by further dideoxy sequencing of two independent plasmid clones for each of the two genes. For each gene, the two independent clones were identical.

The light and heavy chain cDNA inserts of p3F8Kappa.8 and p3F8gamma3.21, respectively, were transferred to mammalian expression vectors. The 3F8 light chain cDNA was inserted into a pMT2PC derivative as described for chimeric light chain cDNA in Example II (see R. Kaufman, Methods in Enzymology 185, 537-566 (1990). The heavy chain cDNA was inserted into pMT3SV₂ADA as described for chimeric heavy chain cDNA in Example II (see R. Kaufman, supra). The plasmid products, p3F8.K2 and p3F8.H1 were

9

used to produce recombinant murine 3F8, in accordance with the present invention. In Example II, these plasmids were used for construction of the mouse/human chimeric 3F8 light and heavy chain genes.

5   The nucleotide and inferred peptide sequences for murine 3F8 light and heavy chain cDNAs are shown in Figure 1a and b, respectively. The complementarity determining regions (CDRs) 1, 2 and 3 of native 3F8 V_L, using the definitions of Kabat et al. [Sequences of Proteins of Immunological Interest, U.S. Dept of

10   Health and Human Services (1987)], are encoded by nucleotides 134-166, 212-232, and 329-346, respectively, of Figure 1a. The CDRs 1, 2 and 3 of native 3F8 V_HDJ, as deduced from comparison with the immunological database of Kabat et al. (supra), are encoded by nucleotides 152-166, 209-256, and 353-385, respective-

15   ly, of Figure 1b.

Example 2 - Construction of DNA Sequences
Encoding Chimeric 3F8-Type Antibodies

       These genes were assembled using conventional recombinant

20   DNA techniques. In the simplest case, primers hybridizing to the 5'-end of the murine V leader region DNA and the 3'-end of the murine J region DNA were designed so that a polymerase chain reaction (PCR), using a murine V(D)J region template, yielded a product with a unique restriction enzyme site at the 5'-end of

25   the gene and a blunt end at the 3'-end of the V(D)J region. Primers hybridizing to the ends of a human constant region template were designed so that the PCR products contained a second unique restriction site at the 3'-end of the constant region and a blunt end at the 5'-end of the constant region.

30   These two PCR-derived gene fragments were then joined with an appropriately restricted eukaryotic expression vector in a tripartite ligation reaction, resulting in a plasmid that contains a chimeric immunoglobulin gene.

TABLE I:　CHIMERIC 3F8 CONSTRUCT SUMMARY

Product		Fragments in ligation	PCR		
Chimeric	Plasmid		Template	5' Primer	3' Primer
A. 3F8 V_LJ-human C_K (c-3F8L)	p8.17	1) 3F8 V_LJ	p3F8Kappa.8	3F8KECO	3F8KBLU
		2) human C_K	pKAP3.1	HUKBLU	HUKSAL
		3) pMT2PCVIIIx.DHFR E/S vector fragment			
B. 3F8 V_HDJ-human C_{XI} (3F8H1)	p1P-1D	1) 3F8 V_HDJ	p3F8gamma3.21	3F8GECO	3F8GBLU
		2) human C_{xl}	pF8/1H.18 cDNA	HUG1BLU	CDNAG 13
		3) E/S pMT35V_2ADA vector fragment			
C. 3F8 V_LJ-human C_K with introns (g-3F8L)	1) pxx3	a) X81 leader-leader intron	pF8/1L.3	K95PCR	3F8V3BLU
		b) 3F8 V_L to KpnI	p8.17	3F8MTBLU	3F8MTKPN
		c) E/K p8.17 vector fragment			
	2) pVK4	a) KpnI to end of 3F8 V_L	p8.17	3F8V3KPN	3F8V3BLU
		b) X81 J, chimeric J/C intron, human C_K and 3' untranslated	pF8/1L.3	F8K1NBLU	KAP3SAL
	3) pVK4-9	a) X/S pVK4 vector fragment			
		b) X/S pF8/1L.3 insert			

Product

Chimeric	Plasmid	Fragments in ligation	PCR Template	5' Primer	3' Primer
D. 3F8V_HDJ-human $c_{\varkappa 2}$ (3F8H2)	p3F8v.hugam 2 .1a. A22	a) 3F8 V_HDJ b) human $C_{\varkappa 2}$ c) E/S MT3SV2ADA vector fragment	p3F8gamma3.21	3F8GECO	3F8GBLU
E. 3F8V_HDJ-human $C_{\varkappa 3}$ w/introns (3F8H3)	1) pVI8	a) 3F8V_HDJ b) 3F8 J intron c) E/S pMT3SV2ADA vector fragment	p1p-10 pF8hv14.3	3F8GECO 3F8GBLU2 F8HINBLU G1THREE	
	2) pY8.15	a) $C_{\varkappa 3}$ b) s pVI8			
F. 3F8 V_HDJ-human $C_{\varkappa 4}$	pZ8.12	a) $C_{\varkappa 4}$ b) s pVI8			

12

A. Construction of Plasmid p8.17: Chimeric 3F8 V_LJ-Human C_κ cDNA Hybrid (c-3)

The PCR-derived DNA comprising the 3F8 V_LJ region was prepared as follows (see Table I for summary). The template for the PCR was p3F8kappa.8. The PCR primer used for the 5'-end of the murine V_L region was 3F8KECO (TACGAATTCAAGATGAAGTCACAGACCCA GGT), containing an EcoRI restriction site and nucleotides 9 to 32 which hybridize from four base-pairs upstream of the transla- tion starting codon ATG of the murine V_L leader region to 20 base-pairs within the gene (Figure 1a, base-pairs 1 to 24). The PCR primer for the 3'-end of the J region was 3F8KBLU (CCGTTTTAT- TTCCAGCTTGGTCCCCCCTCC) and corresponds to nucleotides 379 to 350 in Figure 1a). This primer was phosphorylated prior to use. The conditions for all PCRs were as specified by Perkin-Elmer-Cetus or determined empirically by conventional methods.

The PCR-derived DNA for the human C_κ region was prepared as follows (see Table I for summary). The template for the PCR was pKAP3.1, a plasmid containing the human genomic C_κ region. This clone was isolated from the lung fibroblast cell line WI38 and sequenced using conventional methods.

The PCR primer for the 3' end of the human C_κ region was HUKSAL (TAGAGTCGACTCCCTCTAACACTCTCCCCTGTTG), containing a SalI restriction site and nucleotides 10 to 34 corresponding to sequence from 6 base-pairs downstream of the stop codon to 16 base-pairs upstream of the stop codon within the human C_κ gene (Figure 2, nucleotides 711 to 687).

The PCR primer for the 5'-end of the human C_κ region was HUKBLU (ACTGTGGCTGCACCATCTGTCTTCATCTT) and corresponds to nucleotides 385 to 413 in Figure 2. The 5'-end of HUKBLU is the first complete codon of the human C_κ gene. The codon generated endogenously by the J/C splicing mechanism is amino acid R and was contributed by the murine PCR fragment described above. HUKBLU was phosphorylated prior to use in the PCR.

The PCR-derived fragments were digested appropriately with either EcoRI or SalI and purified by standard electrophoretic methods. The expression plasmid chosen for this chimeric gene was derived from pMT2PC (R. Kaufman, _supra_) which contains a SalI

restriction site downstream of the EcoRI site and a DHFR gene.
The plasmid was digested with EcoRI and SalI and purified from
the original insert by electrophoretic methods.   A tripartite
ligation, containing the two PCR-derived gene fragments and the
expression vector fragment, was performed using standard methods.
After conventional transformation and DNA analysis procedures,
one representative clone, p8.17, was chosen to be sequenced. One
copy of the chimeric 3F8 V_LJ-human C_κ cDNA (c-3F8L, Figure 2) was
inserted between the EcoRI and SalI sites of the expression
vector.   Other than a silent mutation in the human C_κ region
(Figure 2, base-pair 444), the sequence was identical to the
contributing 3F8 and human sequences.

B. Construction of Plasmid p1P-10: Chimeric
3F8 V_HDJ-human $C_{\gamma 1}$ cDNA Hybrid (c-3F8H1)

The PCR-derived DNA for the murine V_HDJ region was prepared
as described below (see also Table I). The template for the PCR
was the murine 3F8 $V_HDJC_{\gamma 3}$ plasmid, p3F8gamma3.21 of Example I.
The primer for the 5'-end of the murine V_HDJ region was 3F8GECO
(TACGAATTCGAGCATGGCTGTCCTGGTGCTG), containing an EcoRI restric-
tion site (nucleotides 1-9) and nucleotides 10 to 31 which
correspond to sequence from 4 base-pairs upstream of the
translation starting codon ATG of the murine V_H leader region to
18 base-pairs within the gene (Figure 1b, nucleotides 1 to 22).

The primer for the 3'-end of the J region was 3F8GBLU
(GGAGGAGACGGTGACTGAGGTTCCTT). It hybridizes to the 3'-end of the
J region (Figure 1b, nucleotides 393 to 418), except for a
mismatch at base-pair 418.   The mismatch was incorporated into
the final product and did not change the amino acid sequence.
3F8GBLU was phosphorylated prior to use in the PCR.

The PCR-derived DNA for the human $C_{\gamma 1}$ region was prepared as
follows (see also Table I).   The template was first-strand
synthesis cDNA from an RNA sample. This RNA was isolated from
COS cells that had been transfected with an expression plasmid,
pX81H. 18 which contained an unrelated chimeric V_HDJ-human $C_{\gamma 1}$
gene.

The PCR primer for the 3'- nd of the human $C_{\gamma 1}$ region was

14

CDNAG13 (TCTAGAGTCGACCGGGGCCGTCGCACTCAT), containing a SalI restriction site (nucleotides 1 to 12) and nucleotides 13 to 30 which hybridize to base-pairs 1414 to 1431 (Figure 3) of human $C_{\gamma 1}$ DNA. The PCR primer for the 5'-end of the constant region was

5   HUG1BLU (GCCTCCACCAAGGGCCCATCGGTCT), where nucleotides 2 to 25 hybridize to the 5'-end of human $C_{\gamma 1}$ (base-pairs 426 to 449 as numbered in Figure 3) and nucleotides 1 to 3 are the codon created by joining the 3F8 J and human $C_{\gamma 1}$ splice junctions. This primer was phosphorylated prior to the PCR.

10   The expression vector chosen for this chimeric construct was pMT3SV₂ADA which contains a SalI restriction site downstream of the EcoRI site and an ADA gene (R. Kaufman, supra). The expression vector and PCR-derived fragments were restricted, purified and used for a tripartite ligation as described above.

15   After conventional transformation and DNA analysis procedures were performed, one clone, PT2-10, was chosen to be sequenced. One copy of the chimeric 3F8 V_HDJ-human $C_{\gamma 1}$ cDNA was inserted between the EcoRI and SalI sites of the expression vector. A mutation in the constant region was corrected by conventional

20   recombinant DNA techniques, and the plasmid product was p1P-10. Figure 3 shows the sequence of the chimeric 3F8 V_HDJ-human $C_{\gamma 1}$ cDNA (c-3F8H1).

C. Construction of Plasmid VK4-9: Chimeric 3F8
V_LJ-Human C_κcDNA/Genomic Hybrid (g-3F8L)

25   In the first of three cloning steps, a PCR-derived fragment comprising the leader and leader intron of an unrelated chimeric V_LJ-human C_κ gene, g-X81L, was prepared from plasmid pX81L.3 (this plasmid contains g-X81L inserted into a pMT2PC derivative vector

30   and was constructed using conventional cloning procedures).

       The 5' PCR primer was K95PCR (TACGAATTCCAGAGATGGAGACAGACACA) containing an EcoRI restriction site (nucleotides 1 to 9) and nucleotides 9 to 28 which correspond to the 5' end of the X81L V_L leader region (base-pairs 6 to 25 in Figure 4). The 3' PCR

35   primer was F8LINBLU (ACCAGTGGAACCTGGAATGATAAACAC) which comprises the 3'-end of the V_L leader intron and begins with the last base-pair of the leader sequence (nucleotides 311 to 285 in Figure 4).

15

This primer was phosphorylated prior to using for the PCR.

The second PCR-derived DNA for step 1 contains most of the
3F8 V_L sequences of p8.17 of section A. The 5' PCR primer was
3F8MTBLU (AGTATTGTGATGACCCAGACTCCCAAATTC) and corresponds to
sequence encoding the N-terminus of the mature 3F8L product
(nucleotides 312 to 341 in Figure 4). This primer was phosphory-
lated prior to use in the PCR. The 3' PCR primer was 3F8MTKPN
(CTGTTGGTACCAAGTTACATCATTACTC) and corresponds to sequences
within the 3F8 V_L region, including a unique KpnI site (Figure 4,
nucleotides 425 to 398).

The two PCR products were restricted with the appropriate
enzyme and purified. Plasmid p8.17 was digested with EcoRI and
KpnI and the large vector fragment was purified from the
EcoRI/KpnI fragment encoding a portion of 3F8 V_L (Figure 2, base-
pairs 1 to 178). The vector fragment and the two PCR fragments
were ligated to each other and resulted in the plasmid, pXX3.
This plasmid is similar to p8.17 but contains the leader and
leader intron of X81 V_L (base-pairs 11 to 311 from Figure 4)
instead of the 3F8 V_L leader. The expected sequence for the PCR-
derived fragments of pXX3 was verified by dideoxynucleotide
sequencing.

In the second cloning step, a PCR fragment corresponding to
3F8 V_L sequences downstream from and including the unique KpnI
site was prepared from plasmid p8.17. The 5' PCR primer was
3F8V3KPN (ACTTGGTACCAACAGAAGGCAGGGCAGT) which hybridizes
completely to 3F8 V_L sequence overlapping the unique KpnI site
(base-pairs 411 to 438 in Figure 4). The 3' PCR primer was
3F8V3BLU (GAACGAACTATAATCCTGCTGACAGAAAT). It corresponds to the
3'-end of 3F8 V_L (base-pairs 596 to 568 in Figure 4), starting at
the final nucleotide of this region. This primer was phosphory-
lated prior to use in the PCR.

A second PCR fragment, comprising the X81 J, chimeric J/C
intron, and human C_κ sequences of g-X81L, was derived from
plasmid pX81L.3. The 5' PCR primer was F8KINBLU (GGTGGAGGCACCAA-
GCTGGAAAT CAAAC) which hybridizes to the J region of X81 (base-
pairs 597 to 624 in Figure 4). This primer was phosphorylated
prior to using for the PCR. The 3' PCR primer was KAP3SAL

(CATCCGGTCGACCGGGTCCCCTGTGGAA) containing a SalI restriction site (nucleotides 1 to 12) and nucleotides 15 to 28 which correspond to sequence downstream from the stop codon at the end of the human C_κ gene (nucleotides 1420 to 1407 in Figure 4). The two PCR-derived fragments were ligated with KpnI- and SalI-digested pXX3 (see above) that had been purified from the KpnI to SalI region of the c-3F8L cDNA (base-pairs 174 to 715 in Figure 2). A clone from this ligation and transformation was defined as plasmid pVK4.

In the third cloning step, the DNA between the unique XbaI and SalI site (sequence from within the chimeric J/C intron to downstream of the human C_κ gene; base-pairs 673 to 1428 in Figure 4) was replaced with the same region from pX81.L3 using standard techniques. A clone from this ligation and transformation was designated as plasmid pVK4-9. The expected sequence between the KpnI and XbaI sites of pVK4-9 (415 to 678 in Figure 4) was verified by dideoxynucleotide sequencing.

Plasmid pVK4-9 is comprised of, in addition to the expression vector DNA, a unique EcoR1 site (1-6; numbers in parentheses refer to sequence in Figure 4), the X81 V_L leader and leader intron (11-311), the 3F8 V_L (312-596), the X81 J (597-624) and 52 base-pairs of flanking murine J/C intron (625-676), 336 base-pairs of human J/C intron (677-1012), the adjacent human C_κ gene (1013-1335), 3' untranslated sequence (1336-1422) and a unique SalI site (1423-1428). In addition to the leader, leader intron, J region, and chimeric J/C intron of plasmid pX81L.3, pVK4-9 also contains 82 nucleotides of untranslated sequence that are not present in p8.17. Since the amino acids encoded by the X81 J region are identical to those encoded by the 3F8 J, both p8.17 and pVK4-9 encode the same mature chimeric protein.

D. Construction of Plasmid p3F8v.hugam2.1a.A22: Chimeric 3F8 V_HDJ-Human $C_{\gamma2}$ cDNA Hybrid (c-3F8H2)

A chimeric gene, c-3F8H2, having a human $\gamma2$ constant region was assembled via a tripartite ligation that contained two PCR fragments and a vector fragment. The PCR-derived fragment that comprises 3F8 V_HDJ was as described above.

17

First strand cDNA that contained human $C_{\gamma 2}$ sequence was used as template for a second PCR. The cDNA was prepared from COS RNA that had been isolated from cells transfected with plasmid p2-4. This plasmid contains a chimeric X81 V_HDJ-human $C_{\gamma 2}$ gene that was constructed by standard methodologies. The chimeric contained, in addition to the internal $C_{\gamma 2}$ introns, a murine/human chimeric J/C intron. The human $C_{\gamma 2}$ genomic DNA was isolated from a genomic library of the lung fibroblast cell line, WI38 (Stratagene), and sequenced by conventional methods.

The 5′ PCR primer was HUG2BLU (GCCTCCACCAAGGGCCCATCGGTC). which hybridizes to the 5′-end of the constant region (Figure 5, base-pairs 425 to 448) and begins with the codon created by a joining the murine J and human C splice junctions. This primer was phosphorylated prior to used in the PCR.

The 3′ PCR primer was IG2.PCR (TCTGTCGACTTGCTGGCCGTGGCACTCA TTT) containing a SalI restriction site (nucleotides 1 to 9) and nucleotides 10 to 31 which hybridize to base-pairs 1400 to 1421 (Figure 5) overlapping the termination codon of the human $C_{\gamma 2}$ gene.

The PCR-derived gene fragments were prepared for the ligation step as described above. The pMT3SV$_2$ADA plasmid was digested with EcoRI and SalI, purified by standard electrophoretic methods and combined with the two PCR fragments for a tripartite ligation. Conventional transformation and DNA analysis procedures resulted in the identification of plasmid p3F8v.hugam2.1a.A22. The chimeric 3F8 V_HDJ-human $C_{\gamma 2}$ gene was inserted between the EcoRI and SalI sites of the vector. Figure 5 shows the sequence of this chimeric gene which is identical to the contributing 3F8 and human DNAs. Tetrameric 3F8-type antibodies having the human $C_{\gamma 2}$ isotype may be produced using this plasmid in combination with either p8.17 or pVK4-9, and known expression methods such as those described in Example III.

E. Construction of Plasmids pY8.15 (Chimeric 3F8 V_HDJ-Human $C_{\gamma 3}$ with Introns) and pZ8.12 (Chimeric 3F8 V_HDJ-Human $C_{\gamma 4}$ cDNA/Genomic Hybrids)

Chimeric 3F8 constructs encoding the human γ3 and γ4 constant r gions were generated from 3F8 V_HDJ cDNA and C_γ genomic

fragments. The construction of g-3F8H3 and g-3F8H4 was done in two cloning steps, the first being a common step requiring two PCR fragments. The first PCR fragment, comprising 3F8 V_HDJ, was derived from p1P-10. The 5' PCR primer was 3F8GECO as described

5 above (see Table I). The 3' PCR primer was 3F8GBLU2 (CTGAGGAGAC-GGTGACTGAGGT) which hybridizes to the 3'-end of 3F8 V_HDJ (Figure 1b, base pairs 388 to 419)

The second PCR fragment, consisting of intron sequences adjacent to the X81 J, was derived from plasmid pX81hv14.3, which

10 contains X81 V_HDJ genomic sequences. This genomic DNA was isolated from the X81 hybridoma by standard cloning methodologies. The 5' PCR primer was F8HINBLU (GTAAGAATGGCCTCTCCAGGT) which hybridizes to the first 21 base pairs of the X81 J/C intron (base-pairs 426 to 446 in Figure 6). The 3' PCR primer was

15 G1THREE (TAGAGTCGACGATTAGTCTGCAATGCTCAGAAAAC) containing a SalI restriction site (nucleotides 1 to 10) and nucleotides 11 to 35 which correspond to sequence within the X81 J/C intron (nucleotides 496 to 472 in Figure 6). The PCR-derived fragments were ligated to pMT3SV$_2$ADA that had been digested with EcoRI and SalI.

20 One product of the transformation and DNA analysis was plasmid pVI8.

Prior to the next cloning step, genomic clones comprising the human $C_{\gamma 3}$ and $C_{\gamma 4}$ regions were isolated by standard methodologies from the lung fibroblast cell line, WI38 (Stratagene) and

25 sequenced by conventional methods. SalI sites were then added to both ends of each gene, including upstream human J/C intron and downstream untranslated sequences (for $C_{\gamma 3}$, base-pairs 497 to 2906 in Figure 6; for $C_{\gamma 4}$, base-pairs 497 to 2339).

For the second cloning step, these SalI human $C_{\gamma 3}$ and $C_{\gamma 4}$

30 fragments (derived from plasmids pYA4 and pZA9, respectively) were inserted independently into the SalI site of pVI8 by conventional procedures. Clones were identified which had the C region in the correct orientations and these were designated plasmid pY8.15 (g-3F8H3) and plasmid pZ8.12 (g-3F8H4). The PCR-

35 derived sequence of plasmid pVI8 was demonstrated to be correct when this portion of plasmid pZ8.12 was sequenced. In Figures 6 and 7 are shown the sequences of the 3F8 V_HDJ-human $C_{\gamma 3}$ and 3F8

V_HDJ-human $C_{\gamma4}$ genes, respectively. In each plasmid there was one
copy of the inserted gene. Tetrameric 3F8-type chimeric
antibodies having the human $C_{\gamma3}$ and $C_{\gamma4}$ isotypes may be produced
using these plasmids in combination with either p8.17 or pVK4-9,
5   and known expression methods such as those described in Example
III.

Example 3 - Expression of Recombinant 3F8-Type Antibodies

10   A. Transient Expression of Recombinant
                    3F8 Constructs in COS Cells
The recombinant murine and chimeric 3F8 light and heavy
chain genes described above were co-expressed initially in COS-1
monkey cells (clone M6) using DEAE Dextran, then a sequential
15   DMSO shock treatment and chloroquin incubation.
Murine and chimeric 3F8-type antibodies were detected by two
methods. First, ELISA systems were established to quantitate the
antibodies secreted into conditioned media during a two or three
day post-transfection incubation. The murine 3F8 ELISA included
20   EIA/RIA plates coated with rabbit anti-mouse IgG (H+L) (62.5
ng/well; Zymed #61-6500), murine IgG3 (Southern Biotechnology
Associates, #105) or hybridoma-derived murine 3F8 standard, and
alkaline phosphatase conjugated rabbit anti-mouse IgG3 (gamma3
chain specific, 45 ng/ml, Zymed # 61-0422). The concentration
25   of the standard was normalized by measuring absorbance at A280,
using an extinction coefficient of 1.4, and was then diluted in
DME/10% fetal calf serum and stored as aliquots at -80°C.
Samples of conditioned media were routinely centrifuged to remove
cell debris and aliquots of supernatant then stored at -80°C. A
30   fresh thaw of both the IgG3 standard and conditioned media were
used in a given ELISA. The assay was developed with p-nitrophe-
nyl phosphate, optical densities measured at 405 nm, and the data
analyzed using Softmax version 2.01 (Molecular Devices).
The ELISA system ultimately established for the quantitation
35   of chimeric 3F8 IgG1κ included EIA/RIA plates coated with goat
anti-human Fc (250 ng/well; Jackson ImmunoResearch #109-005-098),
human IgG1 standard from either Chemicon (#AG502) or Calbiochem
(400112), and alkaline-phosphatase conjugated goat anti-human Fc

20

(10 ng/well; Jackson ImmunoResearch #109-055-098). The concen-
tration of the re-solubilized human IgG1 standard was normalized
by measuring absorbance at A280, using an extinction coefficient
of 1.4, and was then diluted in DME/10% fetal calf serum and
5   stored as aliquots at -80°C. Samples of conditioned media were
routinely centrifuged to remove cell debris and aliquots of
supernatant then stored at -80°C. A fresh thaw of both the
standard human IgG1 and conditioned media were used in a given
ELISA and developed and analyzed as indicated above.

10   Recombinant murine and chimeric 3F8-type antibodies were
also detected by metabolically labelling transfected COS cells,
two or three days post transfection, with ^{35}S methionine and then
immunoprecipitating aliquots of cell lysate and conditioned media
with either Sepharose 4B coupled to protein A, protein G or to
15   goat anti-human IgG(H+L) (Zymed). Reduced or nonreduced
immunoprecipitates were then electrophoresed near ^{14}C-labelled and
reduced protein markers on polyacrylamide gels and visualized by
autoradiography subsequent to enhancement.

The production of chimeric 3F8-type antibody by COS-1 cells
20   was improved when the chimeric 3F8 heavy chain was co-transfected
with a different chimeric 3F8 light chain construct (g-3F8L; see
Example II for construction of pVK4-9). In contrast to the c-
3F8L chimeric cDNA, this construct contains the X81 V_L leader and
leader intron, a chimeric J/C intron and 82 base-pairs of
25   additional sequence 3' of the human C_k gene.

Chimeric 3F8 light chain, if expressed in the absence of a
heavy chain, was secreted into the conditioned media of COS cells
at low levels.

30   ## B. Expression of Chimeric 3F8 IgG1κ in CHO cells

A CHO clone with a DHFR deficiency gene, DUKX-B11, was first
transfected with a chimeric 3F8 light chain construct (c-3F8L;
plasmid p8.17) by protoplast fusion. Several subclones were
immediately isolated and further selected sequentially at 0.005,
35   0.02, 0.1 or 0.2, 1.0, 5.0, 25 and 125 μM methotrexate (MTX).
Secretion of light chain was analyzed using a human kappa chain
ELISA established by conventional methods. 3F8 chimeric light

21

chains are secreted at relatively low levels from transfected
pools of CHO cells select d with nucleoside free media.

CHO lines that had been selected at 0.1 or 0.2 μM MTX were
subsequently transfected with a chimeric 3F8 heavy chain
construct (c-3F8H1; plasmid 1P-10) by liposomemediated transfec-
tion. The cells were cultured in selective media for the
expression of the murine ADA gene as described by R. Kaufman et
al. [Proc. Natl. Acad. Sci. USA 83, 3136-3140 (1986)]. The anti-
human Fc ELISA (see above) was used to follow the accumulation
of tetrameric antibody. Gel analysis of conditioned media
samples by either Western and/or metabolic labelling demonstrated
that heavy chain does not appear to be secreted unless associated
with light chain.

After the initial ADA selection, with 0.2 μM MTX and 0.03
μM deoxycoformycin (dCF), chimeric 3F8-type antibody was detected
at 3 μg/2x10^6 cells/24 hrs. CHO lines (CHL3) have been estab-
lished that are stable at high levels of MTX and dCF and certain
ones express chimeric 3F8-type IgG1κ at $\geq$ 80 μg/1x10^6 cells/day
(40 μM MTX/10 μM dCF.

Recombinantly-produced 3F8-type antibody was also obtained
from CHO lines (KH) in which light chain was expressed from a
different construct (g-3F8L; plasmid pVK4-9). Certain amplified
lines produced $\geq$ 80 μg/1 X 10^6 cells/day (10 or 20 μM MTX/7 or 10
μM dCF).

Example 4 - Purification of Recombinant 3F8-Type Antibodies
    A. Purification of Recombinant Murine 3F8-Type Antibody

As an example of the method, conditioned medium containing
recombinant murine 3F8-type antibody is adjusted to pH 5.0 with
glacial acetic acid and loaded onto a column of S-Sepharose
equilibrated in 25mM acetate, 0.15M NaCl, pH5.0. The column is
washed with equilibration buffer then eluted with 25mM acetate,
0.5M NaCl, pH5.0. The eluted pool of partially purified murine
3F8-type antibody is adjusted to 75mM ac tate and 0.5mg/ml total
protein to ensure antibody solubility prior to pH adjustment. It
is then titrated to pH 8.0 using 1M Tris base. This material is
loaded onto a column of Sepharose-Protein A, equilibrated in 50mM

glycine, 1M NaCl, pH 8.8. The column is washed with 50mM glycine, pH 8.8, then eluted with 75mM acetate, 0.13M NaCl, pH 4.0. The eluate is adjusted to a protein concentration of 0.5mg/ml to ensure solubility and is titrated to pH 8.0 with 1M Tris.HCl, pH

5   9.0. This material is loaded onto a column of Q-Sepharose equilibrated in 10mM Tris, 75mM acetate, 0.1M NaCl, pH 8.0. The column is washed with this buffer and the flow-through, which contains murine 3F8-type antibody, is pooled. The pool is adjusted to pH 5.0 with glacial acetic acid and loaded onto a

10   column of S-Sepharose equilibrated in 75mM acetate, 0.1M NaCl, pH 5.0. The column is washed with equilibration buffer, then with 50mM sodium phosphate, pH 6.0 before being eluted with 50mM sodium phosphate, 0.5M NaCl, pH 6.0. Fractions containing 3F8 are pooled.

15

B. Purification of Chimeric 3F8-Type IgG1κ

The chimeric 3F8-type antibody of the present invention binds quite well to protein A resin but could not be eluted under standard conditions that should not denature the antibody. It

20   was necessary therefore to use other conventional chromatographic resins for the purification of chimeric 3F8-type antibody from defined conditioned media.

Since chimeric 3F8-type IgG1κ has a relatively high pI, MonoS FPLC was chosen as the first purification step. Defined

25   conditioned media was diluted two or three fold with 20 mM MES pH 5.5, the pH dropped to 5.5 with 1 N HCl, and then filtered through a Nalgene 0.2 μm membrane. Under these conditions, chimeric 3F8-type IgG1κ was eluted with a 0-1 M NaCl, 20mM MES pH 5.5 gradient. Coomassie stains of SDS-acryamide gels, reduced

30   and nonreduced, indicated that all major bands represent chimeric 3F8-type antibody.

Example 5 - Characteristics of Chimeric 3F8-type IgG1κ Antibody

The chimeric 3F8-type IgG1κ antibody was stable to degrada-

35   tion based on the Western and ELISA analysis of heavy and light chain presence in complete and defined conditioned media, even after storage at 4°C for several weeks.

23

Unreduced samples of chimeric 3F8-type antibody migrated near the boundary betw en the stack and separating gels of a 10% polyacrylamide-SDS gel, in agreement with the reported observations for immunoglobulin molecules assayed on this type of gel
5   [S. Fasler et al., Anal. Biochem. 174, 593-600 (1988)]. The apparent sizes of the chimeric light and heavy chains are approximately 27 and 50 kd respectfully (reduced samples analyzed by SDS-10% PAGE). The pI for chimeric 3F8 IgG1κ is approximately 8.5.

10   The ganglioside specificity of the chimeric 3F8-type IgG1κ antibody, as determined by TLC-immunostaining methods, was similar to that of murine mab 3F8 derived from the hybridoma. Nano-HPTLC plates, pre-coated with silica gel 60, were loaded with a panel of gangliosides (GQ1b, GT1b, GD1b, GD2, GD1a, GD3,
15   GM1, GM2 and GM3 at 1 nmole sialic acid; Biocarb) and chromatographed in chloroform/ methanol/ water/CaCl$_2$. Plates were then incubated in PBS containing 0.3% gelatin (wash buffer) for 1-2 hrs, then incubated for 2-4 hrs with 5 μg/ml of either murine or chimeric 3F8-type antibody (diluted in media that contains PMSF
20   and soybean trypsin inhibitor). The plates were washed briefly with wash buffer and then incubated for 2-4 hours with 4-7 μCi of ^{125}I-protein A in wash buffer. The plates were washed in PBS containing 0.1% TritonX, dried and exposed to film at -80°C. Gangliosides on a separate HPTLC plate, which had been chromato-
25   graphed with those immunostained, were visualized by charring.

Both the murine 3F8-type IgG3κ and chimeric 3F8-type IgG1κ antibodies reacted strongly with only ganglioside GD2. No cross-reactivity in other ganglioside lanes was observed with the exception of faint. spots that co-migrated with the ganglioside
30   GD2 spot.

As assayed in a chromium release assay, chimeric 3F8-type IgG1κ antibody mediated the killing of tumour target cells by peripheral blood lymphocytes (PBL) similar to murine 3F8 IgG3κ derived from the hybridoma. In particular, 2 x 10^6 SK-MEL-1
35   (ATCC) human melanoma cells wer   incubated with 100 μCi [51]Cr (420 mCi/mg sodium chromate; New England Nuclear) for 1 hour at 37°C in 500 μl fetal calf serum (FCS). The radiolabelled cells were

24

washed once, resuspended in 10 ml RPMI 1640 (Hazleton Biologics, Inc.), 10% FCS and incubated for 1 hour at 37°C. The cells were then washed once and resuspended in RPMI 1640, 10% FCS and plated at 1 x 10⁴ cells/ well (100 μl) in 96 well, round bottom, tissue culture plates (Costar)

Frozen aliquots of conditioned media containing chimeric 3F8 IgG1κ [concentration determined by an anti-human Fc ELISA (see example III)] were thawed, diluted in triplicate in RPMI 1640, 10% FCS and 50 μl of each dilution added to a well with target cells.

Leukopaks (4-20 mls), which had been stored at 4°C for approximately 16 hrs, were diluted to 80 mls and 20 ml aliquots layered over Ficoll-Hypaque (Pharmacia) and centrifuged at 1800 rpm, 4°C, for 30 min. The cellular interface was isolated and then washed and resuspended in RPMI 1640, 10% FCS. The cells were then added to T175 cm² tissue culture flasks (Falcon, Becton Dickinson Labware) that have been previously coated with 10% human plasma in RPMI 1640 for 1 hour at 37°C. The PBMC (peripheral blood mononuclear cells) were allowed to adhere to the coated plastic for 1 hour at 37°C. All non-adherent PBL were removed and washed one time with RPMI 1640, 10% FCS. The PBL were plated at 5 x 10⁵ cells/well (in 50 μl) into wells that already contained target cells and antibody. The PBL effector:target ratio of the assay was 50:1. The 96 well microtiter plates were incubated for 4 hours at 37°C in a humidified atmosphere of 5.0% CO₂. Then, 100 μl of each supernatant was removed, and the radioactivity determined using a Beckman Gamma Counter (Beckman Scientific Instruments Inc.).

The cell-mediated killing of the target cells was calculated as follows:

$$\text{percent lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total release cpm} - \text{spontaneous cpm}} \times 100$$

Spontaneous cpm corresponds to the radioactivity released from target cells in the absence of effector cells or antibody. The addition of effector cells without antibody did not cause any additional lysis, thus percent lysis reflects antibody mediated lysis by PBL effectors. Total release cpm represents the

25

radioactivity released from target cells (100 μl) treated with
1% TritonX-100 (100 μl) during the assay incubation period.

Initial determinations indicated that the 50% maximal lysis
for two samples of chimeric 3F8 IgG1κ were 8.9 $\pm$ 0.7 ng/ml and
4.5 $\pm$ 2.4 ng/ml, respectively. Comparable results were obtained
with purified chimeric 3F8 IgG1κ. Chimeric 3F8 IgG1κ also
mediates the killing of the neuroblastoma LAN-1 and NMB-7 cell
lines.

As assayed in a chromium release assay, chimeric 3F8 IgG1κ
antibody also mediates the killing of melanoma tumour cells by
human complement similar to murine 3F8 IgG3κ derived from the
hybridoma. SK-MEL-1 and antibody samples were prepared and
aliquotted as indicated above. Aliquots of human serum, stored
at -80°C, were thawed and used as the source of complement.
Complement was added (50 μl) to a well with target cells and
antibody (150 μl) such that the final titer of the complement was
1:4. Microtiter plates containing the target cells, antibody
dilutions and human complement were incubated at 37°C for 3 hours
in a humidified atmosphere of 5.0% CO_2. Then, 100 μl of each
supernatant was harvested and the radioactivity detected using
a gamma counter.

The complement-mediated killing of the target cells was
calculated as follows:

percent lysis = $\dfrac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total release cpm} - \text{spontaneous cpm}}$ x 100

Spontaneous cpm corresponds to the radioactivity released from
target cells in the absence of complement or antibody. In some
instances, there was antibody independent killing by complement
and this was reflected in the baseline of the antibody dilution
curve at low antibody concentrations. Total release cpm
represents the radioactivity released from target cells by
treating target cells (100 μl) with 1% triton X-100 (100 μl)
during the assay. Initial determinations of the 50% maximal
lysis for two samples of conditioned media were 337 $\pm$ 107 ng/ml
and 213 $\pm$ 60 ng/ml. Comparable results were obtained with
purified chimeric 3F8 IgG1κ.

Chimeric 3F8 IgG1κ also augments/m diates the phagocytosis
of melanoma and neuroblastoma target cells by monocytes that have

been cultured in the presence of M-CSF, using the methods of D. Munn and N.K.V. Cheung, J. Exp. Med. <u>170</u>:511-52 (1989) and <u>172</u>:231-237 (1990). It also mediates the killing of melanoma and neuroblastoma target cells by granulocytes.

5

CLAIMS

1.   A recombinantly produced 3F8-type antibody substantially free from murine proteins.

2.   A composition comprising a DNA sequence selected from the group consisting of:

    (i) a DNA sequence containing a sequence selected from the group consisting of the sequences of Figures 1a, 2 and 4; and

    (ii) a DNA sequence differing from the DNA of (i) in codon sequence due to the degeneracy of the genetic code.

3.   A composition comprising a DNA sequence selected from the group consisting of:

    (i) a DNA sequence selected from the group consisting of the sequences of Figures 1b, 3, 5, 6, and 7; and

    (ii) a DNA differing from the DNA of (i) in codon sequence due to the degeneracy of the genetic code.

4.   A composition comprising a DNA sequence selected from the group consisting of:

    (i) nucleotides 134-166, 212-232, and 329-346 of Figure 1a, and nucleotides 152-166, 209-256, and 353-385 of Figure 1b; and

    (ii) a DNA differing from the DNA of (i) in codon sequence due to the degeneracy of the genetic code.

5.   A composition comprising a DNA sequence selected from the group consisting of:

    (i) nucleotides 134-166 of Figure 1a;

    (ii)   nucleotides 212-232 of Figure 1a;

    (iii) nucleotides 209-256 of Figure 1b;

    (iv) nucleotides 353-385 of Figure 1b; and

    (v) a DNA differing from the DNAs of (i) through (iv) in codon sequence due to the degeneracy of the genetic code.

28

6. A recombinantly produced 3F8-type chimeric antibody characterized by:

(a) a light chain containing one or more amino acid sequences encoded by nucleotides 134-166, 212-232, or 329-346 of Figure 1a; and

(b) a heavy chain containing one or more amino acid sequences encoded by nucleotides 152-166, 209-256, or 353-385 of Figure 1b.

(c) the absence of nucleotides 380-746 of Figure 1a and nucleotides 422-1462 of Figure 1b which encode the murine constant regions;

(d) binding to the disialoganglioside antigen G_{D2}; and

(e) the absence of substantial binding to the disialoganglioside antigen G_{D3}.

7. A process for producing recombinant 3F8-type antibodies which comprises:

(a) culturing in a suitable culture medium a mammalian host transformed with

(i) a light chain encoding DNA sequence of Figure 1a, 2, or 4, said DNA sequence being in operative association with a first expression control sequence; and

(ii) a heavy chain encoding DNA sequence of Figure 1b, 3, 5, 6, or 7, said DNA sequence being in operative association with a second expression control sequence;

(b) isolating from said host and medium a tetrameric 3F8-type antibody.

8. A process for producing a 3F8-type antibody which comprises:

(a) culturing in a suitable culture medium a mammalian host transformed with

(i) a light-chain-encoding DNA sequence containing nucleotides 134-166, 212-232, or 329-346 of Figure 1a, said DNA sequence being in operative association with a first expression control sequence; and

(ii) a heavy-chain-encoding DNA sequence containing nucleotides 152-166, 209-256, or 353-385 of Figure 1b said DNA

sequence being in operative association with a second expression control sequ nce;

(b) isolating from said host and medium a tetrameric 3F8-type antibody.

9. The process of claim 7 wherein the light chain encoding DNA sequence is that of Figure 1a and the heavy chain encoding DNA sequence is that of Figure 1b.

10. The process of claim 7 wherein the light chain encoding DNA sequence is that of Figure 2 and the heavy chain encoding DNA sequence is selected from the group consisting of the sequences of Figures 3, 5, 6, and 7.

11. The process of claim 7 wherein the light chain encoding DNA sequence is that of Figure 4 and the heavy chain encoding DNA sequence is selected from the group consisting of the sequences of Figures 3, 5, 6 and 7.

12. A pharmaceutical composition for the treatment of cancer comprising a therapeutically effective amount of a recombinant 3F8-type antibody of claim 1 in a parenterally acceptable vehicle.

13. A pharmaceutical composition for the treatment of cancer comprising a therapeutically effective amount of a recombinant 3F8-type antibody of claim 6 in a parenterally acceptable vehicle.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]
According to International Patent Classification (IPC) or to both National Classification and IPC
Int.Cl. 5 C12N15/13;   C07K15/28;   A61K39/395

II. FIELDS SEARCHED

Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category°	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	NUCL. MED. BIOL.; INT. J. RADIAT. APLL. INSTRUM. PART B vol. 16, no. 2, 1989, MARSH BARTON, EXETER, GB; N.-K. V. CHEUNG ET AL.: 'Targeted radiotherapy and immuntherapy of human neuroblastoma with GD2 specific monoclonal antibodies' see page 111, right column, line 16 - page 119, right column, line 50	1,12
X	EP,A,0 295 305 (MEIJI MILK PRODUCTS COMP. LIMITED) 21 December 1988 see claims 1-10; table 5	1,12

° Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

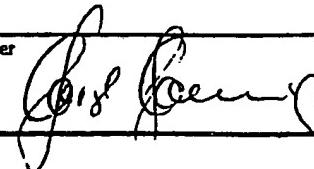| Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 SEPTEMBER 1992	
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	HORNIG H.

Form PCT/ISA/210 (second sheet) (January 1985)

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:   2-11, 13
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

   Figures not present. They are absolutely necessary for executing the search
   for the claims 2-11,13, according to Article 17 (2)/a)(ii)PCT

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all
   searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
   ☐ The additional search fees were accompanied by the applicant's protest.

   ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 11/09/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0295305	21-12-88	WO-A- 8802006	24-03-88